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A brominated-fluorene insect neuropeptide analog exhibits pyrokinin/PBAN-specific toxicity for adult females of the tobacco budworm moth

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Abstract

An analog of the insect pyrokinin/PBAN class of neuropeptides, which features a 2-amino7-bromofluorene attached to the carboxy-terminal bioactive core of the insect pyrokinin/PBAN class of neuropeptides (Phe-Thr-Pro-Arg-Leu-NH_2), via a succinnic acid linker, was tested in adult *H. virescens* moths. This analog was found to induce pheromone production when injected into or applied topically to moths. Topical application of as much as 1 nmol of the analog to moths induced production of significant amounts of pheromone for only 1–2 h, whereas injection of 500 pmol induced pheromone production for up to 20 h. All insects died within 24 h after injection of 500 pmol of the analog. Mortality studies indicated that the LD_{50} for the analog was 0.7 pmol when injected. A non-pyrokinin/PBAN peptide analog formed by attachment of 2-amino-7-bromofluorene to Ala-Ala-Arg-Ala-Ala-NH_2 (via the succinnic acid linker) did not induce mortality when injected at 1 nmol. Similarly no mortality was found when up to 2 nmol of an analog containing a non-brominated fluorene ring, formed by attachment of 9-fluoreneacetic acid to Phe-Thr-Pro-Arg-Leu-NH_2, was injected into moths. The data indicated that both the bromine and active core of the pyrokinin neuropeptides (Phe-Thr-Pro-Arg-Leu-NH_2) were critical for a specific toxic action and suggested that the brominated analog poisoned the moths by interacting with pyrokinin receptors. © 2002 Published by Elsevier Science Inc.

Keywords: Insect neuropeptides; Pyrokinin; PBAN; Pheromone production; Toxicity

1. Introduction

Current awareness of environmental and human health risks associated with use of classic insecticides has resulted in the removal of many compounds from use. Consequently, it is of critical importance that alternative strategies for pest control be developed. Insect neuropeptides are highly attractive candidates for use in the development of new strategies for insect control because they regulate virtually all aspects of insect growth, development and homeostasis [7,11,12]. However, direct use of neuropeptides for insect control is impractical because the insect cuticle contains an apolar lipid matrix that inhibits penetration of polar compounds like peptides and because the insect gut, hemolymph and membranes of a number of tissues contain peptidases that rapidly degrade the peptides [20]. Nonetheless, ways to harness the physiological effects of insect neuropeptides for use in control of insect pests have been proposed [7,8,11, 12]. One such way is development of antagonists or agonists that act at the neuropeptide receptor sites [20]. Design of analogs of neuropeptides that penetrate the cuticle and resist enzymatic degradation is a key step in development of control techniques employing analogs of insect neuropeptides.

We have been developing analogs of neuropeptides that penetrate the insect cuticle for some time using the insect pyrokinin/PBAN class of neuropeptides as models for analog development [20]. This class of neuropeptides regulates numerous physiological events in insects including hindgut and oviduct myotropic activity in cockroaches and locusts, pupariation in flies, induction of egg diapause, reddish coloration and melanization in moths, and sex pheromone biosynthesis in moths and some flies [15,17]. These neuropeptides range in length from 8–34 amino acids but they all share the common C-terminal pentapeptide Phe-Xxx-Pro-Arg-Leu-NH_2 (Xxx = Gly, Ser, Thr or Val). This common sequence forms a type 1 β-turn required for receptor recognition and is the critical portion of these peptides required for bioactivity in all physiological assays [2,12–15]. Studies have demonstrated that the most potent

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of these pentapeptides in pheromonotropic assays is Phe-Thr-Pro-Arg-Leu-NH_2 [17]. Our approach to design of analogs of the pyrokinins has been to attach lipid moieties to the amino terminus of the pentapeptide Phe-Thr-Pro-Arg-Leu-NH_2. Attachment of the lipid moiety imparts an amphiphilic character to the analogs, making them both soluble in water and capable of penetrating the hydrophobic insect cuticle [1,13,15,18,19]. Additionally, these analogs give the active neuropeptide core a degree of resistance to aminopeptidases because the cleavage site of these enzymes is blocked by the lipidic component. The following reports results of studies conducted with one such analog featuring the attachment of 2-amino-7-bromofluorene to the C-terminal active peptide core (Phe-Thr-Pro-Arg-Leu-NH_2) of the insect pyrokinin class of neuropeptides. The results demonstrated that the brominated analog induced adult female tobacco budworm moths to produce pheromone over prolonged periods and also exhibited toxic effects when injected into females.

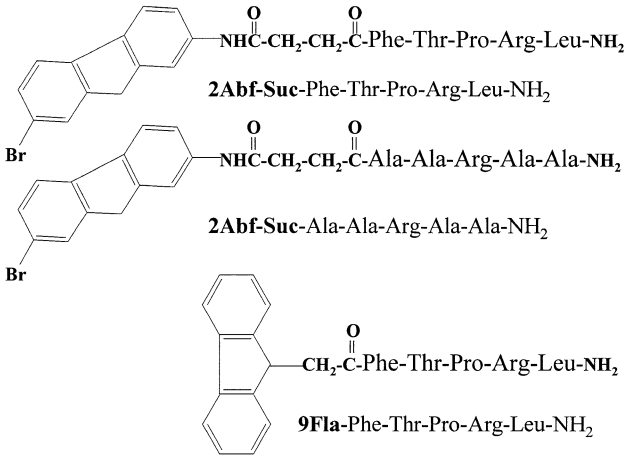

Fig. 1. Structures of pseudopeptides. Names of compounds are below the structures.

2. Materials and methods

2.1. Pseudopeptide synthesis and purification

Pheromone biosynthesis activating neuropeptide was synthesized and purified as described elsewhere [4]. 2-Amino-7-bromo-fluorene (2Abf) and succinnic acid (Suc) were purchased from Aldrich Chemical (Milwaukee, WI). The synthesis of analogs **2Abf-Suc**-Phe-Thr-Pro-Arg-Leu-NH_2 [13,14] and **9Fla**-Phe-Thr-Pro-Arg-Leu-NH2 [13,14,18] have been reported previously. Synthesis of the non-brominated, non-pyrokinin analog **2Abf-Suc**-Ala-Ala-Arg-Ala-Ala-NH_2 was accomplished in stepwise fashion. Ala-Ala-Arg-(PMC)-Ala-Ala-rink amide resin complex was synthesized using FMOC methodology on an ABI 433A peptide synthesizer (Foster City, CA) according to previously described procedures [15]. The pseudopeptide analog was synthesized by condensation of succinnic acid and 2Abf to the Ala-Ala-Arg-(PMC)-Ala-Ala-rink amide resin complex by stirring with one equivalent of 1,3-diisopropylcarbodiimide/1-hydroxy-7-azabenzotriazole in dimethyl sulfoxide for 4 h at room temperature for each step. The crude pseudopeptide was cleaved from the resin and protecting groups were removed by treatment with a mixture of trifluoroacetic acid (TFA) (90%), anisole (5%), thioanisole (4%) and 1,2-ethanedithiol (1%) for 1h. The resin was removed by filtration and volatile reagents were removed by vacuum concentration with a Savant Speed Vac® concentrator. For greater detail consult the previously reported synthetic steps used in preparation of **2Abf-Suc**-Phe-Thr-Pro-Arg-Leu-NH_2 [13,14,16,18]. The crude product was purified on a Waters C_{18} Sep Pak cartridge and a Delta Pak C_{18} reverse-phase column (8 × 100 mm, 15 μm particle size, 100 A pore size) on a Waters 510 HPLC controlled with a Millennium 2010 chromatography manager system (Waters, Milford, MA) with detection at 214 nm at ambient temperature. Solvent A = 0.1% aqueous trifluoroacetic acid (TFA); Solvent B = 80% aqueous acetonitrile containing 0.1% TFA. Conditions: Initial solvent consisting of 20% B was followed by the Waters linear program to 100% B over 40 min; flow rate, 2 ml/min. Delta-Pak C-18 retention time for **2Abf-Suc**-Ala-Ala-Arg-Ala-Ala-NH_2: 16.5 min. The identity of the peptide analog was confirmed via MALDI-MS on a Kratos Kompact Probe MALDI-MS instrument (Kratos Analytical, Ltd., Manchester, UK) with the presence of the following molecular ions (MH^+): 799.2 and 801.2. Quantification was accomplished by amino acid analysis after hydrolysis in 6N HCl using an Applied Biosystems 420A7 amino acid analyzer and led to the following ratios: A[4.0], R[1.0]. Structures of the peptide analogs used in this study are presented in Fig. 1.

2.2. Assay of biological activity of 2Abf-Suc-Phe-Thr-Pro-Arg-Leu-NH_2

Pupae of the moth *Heliothis virescens* were obtained from a laboratory culture maintained at the Center for Medical, Agricultural and Veterinary Entomology, USDA, Gainesville Fl. and were separated by sex with the males being discarded. Females were allowed to eclose to the adult stage in 4-liter containers held in environmental chambers at 25°∀2°, 65%∀3° relative humidity with a 14h:10h light: dark photoperiod. Newly eclosed adults were transferred to new containers daily and provided with a 5% sucrose solution as food. All bioassays were conducted using 3-day old females during the photophase, when endogenous levels of pheromone are low or undetectable [2].

Initially, we assessed the effect of the pseudopeptide in inducing sex pheromone biosynthesis by injecting it into females and comparing the amounts of pheromone produced to amounts produced when females were injected with the optimum dose of PBAN for stimulation of phero-

mone production (5 pmol/20 μl, saline) [1] or just saline. The pseudopeptide was dissolved in saline [4] at various concentrations from 0.1–1000.0 pmol/20 μl and injected into the side of the abdomen. After an incubation period of 1h the terminal abdominal segments, which contain the sex pheromone gland, were excised from the insects and extracted in 20 μl of hexane containing 1 ng/μl of heptadecane and nonadecane as internal standards. The amount of pheromone in individual extracts was determined by quantifying the amount of (Z)-11-hexadecenal (Z11–16:AL) using capillary gas chromatography as described elsewhere [1]. The amount of pheromone in extracts obtained from insects injected with the pseudopeptide or with saline only were converted to a percentage of the mean amount present in extracts obtained from females injected with 5 pmol of PBAN for that day. Data were analyzed using a one-way ANOVA and Tukey = s test using Statmost7 software (DataMost Corporation).

For topical application studies we removed the scales on the surface of the abdomen by gently dabbing the ventral surface of the abdomen on cellulose adhesive tape. Moths were held immobile, ventral side up, by clamping the wings behind the back using smooth jawed alligator clips held in modeling clay. A 1 μl drop of H_2O containing between 1–1000 pmol of the analog was applied to the de-scaled portion of the abdomen [2] and the insects were incubated for 1 h. After incubation the pheromone glands were excised, extracted, and the extracts analyzed as above. Control treatments for this experiment included abdomens treated with only water and females injected with 5 pmol of PBAN and incubated for 1h prior to excision and extraction of the pheromone glands.

Temporal activity studies were conducted by either injecting 500 pmol of pseudopeptide in 20 μl of saline or applying 1000 pmol in a 1 μl drop of H_2O of the pseudopeptide to the abdomen in a 1 μl drop of H_2O or just H_2O. For incubations of up to 12 h applications were made to different groups of moths at 1 h intervals throughout the photophase with the final application being made 1h prior to excision and extraction of the pheromone glands. When incubations of 18–24 h were conducted, insects were treated 6 h after initiation of the photophase and the glands were excised 18–24 h later. Data were analyzed using a one-way ANOVA and Tukey = s test.

Mortality studies were conducted by injecting 10 μl of saline containing different amounts of the analog into female insects. The insects were observed and death was indicated by flaccid mushy abdomens, an inability to respond to touch, and absence of pigment in eyes at 1,2,4,8,12,16,20 and 24 h after injection. For these studies an equal number of insects were injected with either a single dose of peptide or 10 μl of saline (control) on each day. Mortality curves were calculated using GraphPad Prism[tm] dose response software.

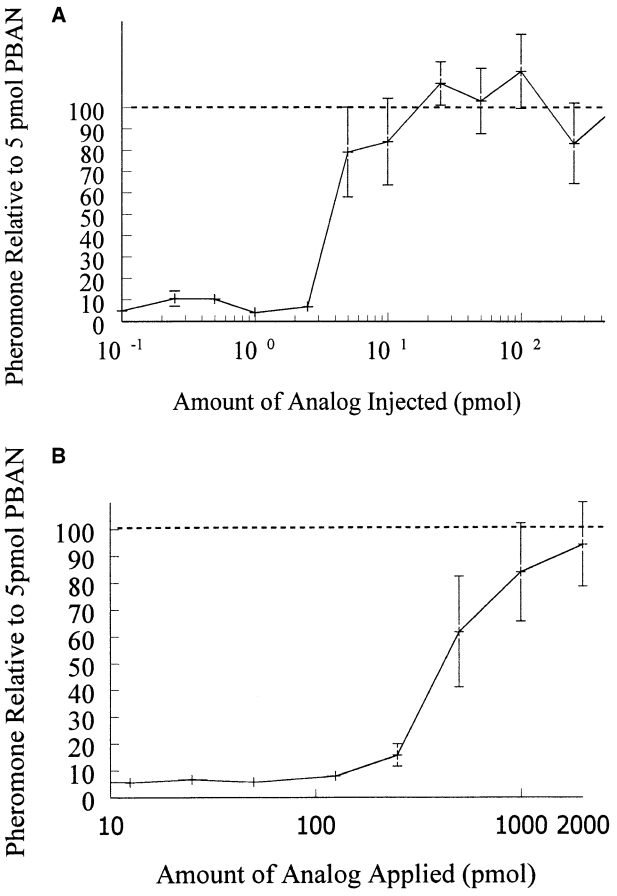

Fig. 2. Dose response curves for showing amounts of pheromone present in extracts relative to that induced by injection of 5 pmol of PBAN (100%, dashed line). Insects were incubated for 1 h after treatments. 2a: Relative amounts recovered after injection of **2Abf-Suc**-Phe-Thr-Pro-Arg-Leu-NH_2 (n = 8/dose, mean ± SE). 2b: Relative amounts recovered after topical application of **2Abf-Suc**-Phe-Thr-Pro-Arg-Leu-NH_2 (n = 8/dose, mean ± SE).

3. Results and discussion

3.1. Induction of pheromone production

Studies conducted to determine if the brominated analog (**2Abf-Suc**-Phe-Thr-Pro-Arg-Leu-NH_2) would stimulate pheromone production when injected into moths indicated that the compound had pheromonotropic properties (Fig. 2). The EC_{50} was determined to be 4.3 pmol and maximum response (that equivalent to 5 pmol of PBAN, EC_{100}) was 10 pmol. These values were more than an order of magnitude greater than those determined from studies in which the non-brominated analog, formed by attachment of 9-floureneacetic acid to Phe-Thr-Pro-Arg-Leu-NH_2 (**9Fla**-Phe-Thr-Pro-Arg-Leu-NH_2, Fig 1) were used [18]. Application of the brominated analog to the cuticle of moths also induced production of pheromone (EC_{50} = 425 pmol; EC_{100} = 1000 pmol) in 1 h but significantly more of the analog was required to induce production of as much pheromone as was induced by injection of 5 pmol of PBAN than when this

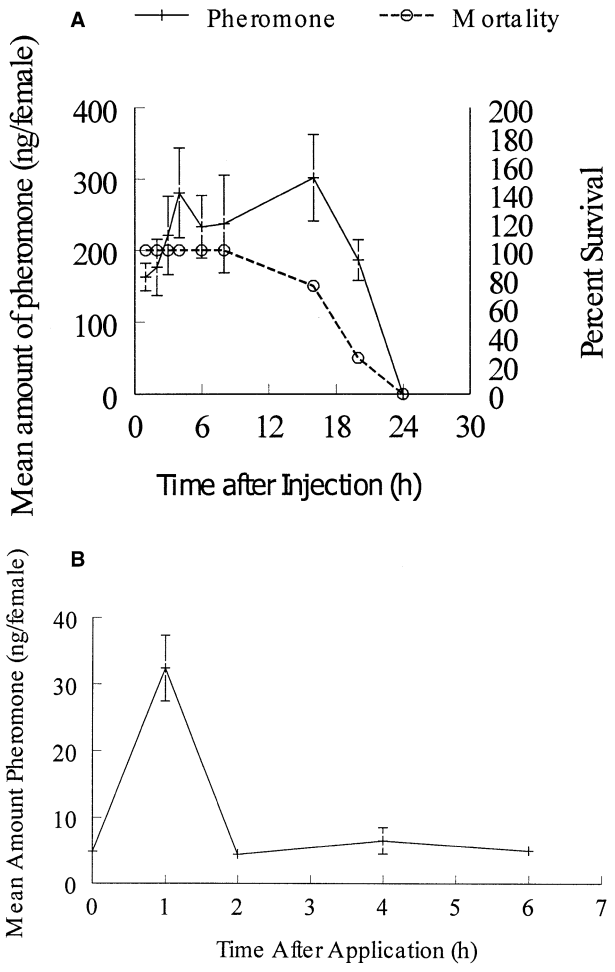

Fig. 3. Effect of time after treatment with **2Abf-Suc**-Phe-Thr-Pro-Arg-Leu-NH_2 on pheromone production (ng/female). 3a: Effect of injection of 500 pmol on both pheromone production and on mortality, as indicated by percent survival (n = 16/dose, mean ± SE). 3b: Effect of topical application of 1000 pmol on pheromone production (n = 8/treatment, mean ± SE).

analog was injected (Fig. 2). Interestingly, the calculated EC_{50} and EC_{100} (EC_{50} = 425 pmol; EC_{100} = 1000 pmol) for the brominated analog, when applied topically, were similar to those values calculated for topically applied 9-fluoreneacetic acid analog (EC_{50} = 525 pmol, EC_{100} = 1000 pmol, data from 18). Thus, although attaching the halogen to the fluorene ring (and different ring attachment site) had a significant negative effect on potency of the peptide analog when injected, it had no effect when applied topically.

Temporal response studies showed that injection of 500 pmol of the brominated analog induced pheromone production for as long as 20 h (Fig. 3). However, at 16 h after injection, 25% of the moths were dead. Seventy-five percent of moths incubated for 20 h after injection died, although those that survived were still producing significant amounts of pheromone. No females survived for 24 h after injection of 500 pmol of the analog. However, females injected with saline alone all survived for 24 h after injection (n = 16). Temporal response studies in which we applied topically

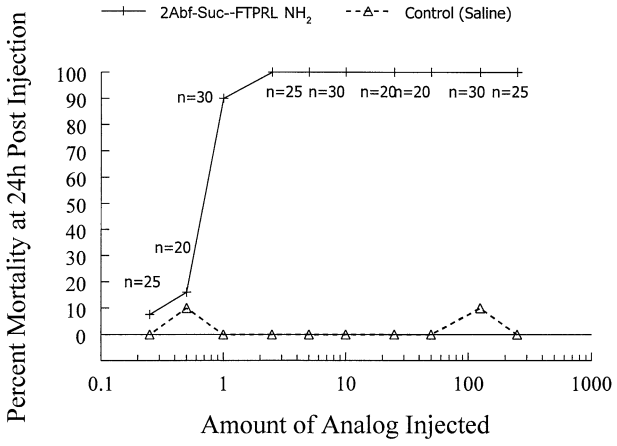

Fig. 4. Effect of injection of different amounts of **2Abf-Suc**-Phe-Thr-Pro-Arg-Leu-NH_2 on mortality 24 h after treatment. Equal numbers of females were injected with either saline (control) or **2Abf-Suc**-Phe-Thr-Pro-Arg-Leu-NH_2 on a given day. Numbers of insects treated are shown for the curve representing **2Abf-Suc**-Phe-Thr-Pro-Arg-Leu-NH_2.

1000 pmol of analog and sampled insects at hourly intervals showed that the pheromonotropic effect of the analog lasted only 1–2h (Fig. 3). This was significantly less than that induced by application of the non-brominaled analog (9Fla-Phe-Thr-Pro-Arg-Leu-NH_2), which elicited pheromonotropic activity for at least 20 h after application [18].

3.2. Mortality studies

The fact that females injected with 500 pmol of the brominated analog were all dead 24h after injection caused us to conduct additional studies to determine the toxicity of the analog. Results of dose response studies, in which we injected the analog, revealed that the compound had toxicological effects (Fig. 4). The LC_{50} was calculated to be 0.7 (0.49–0.93) pmol (R^2 = 0.83). Observation of insects injected with 500 pmol of the brominated analog showed that toxic effects of the analog were manifest by at least 16 h after injection. After 16 h moths were sluggish and did not attempt escape, either by ambulation or flight, when prodded with forceps or when picked up where as saline injected controls invariably attempted escape. Additionally, at 16 h after injection, ca. 50% of treated insects had dark eyes even though the moths were in full light, indicting that pigment in the corneal pigment cells was concentrated near the lens of the eye and had failed to migrate. In saline treated controls the pigment was distributed evenly thorough out the corneal pigment cell. This gave the eye a metallic light green appearance during the day. Dead insects all presented the same symptoms. The abdomens were somewhat distended, flaccid and mushy, the eyes were dark, and the legs and wings were immobile as if rigor mortis had set in. The major differences between females that died after injection with the bromine analog and those that died naturally were that females that died of natural causes routinely deposited numerous unfertilized eggs prior to death, and featured con-

tracted abdomens that were relatively stiff, as though the muscles had contracted.

To determine what was responsible for the mortality we reexamined data that we obtained from studies in which we injected the non-brominated analog, **9Fla**-Phe-Thr-Pro-Arg-Leu-NH_2 [18–20]. Interestingly, no mortality occurred among insects injected with as much as 1 nmol of the non-brominated analog after 24 h. Thus, the bromine (and perhaps alternate ring attachment site) imparted the toxicity to the peptide analog. If this were so then we reasoned that perhaps any peptide having a brominated fluorene ring might be toxic. A non-pyrokinin analog was synthesized that featured retention of the highly charged residue Arg, imparting the same amphiphilic, water-soluble properties observed in the toxic bromine-containing analog. However, when we injected as much as 1000 pmol of the non-pyrokinin analog containing 2-Amino-7-bromo-fluorene as a lipidic component (**2Abf-Suc**-Ala-Ala-Arg-Ala-Ala-NH_2, Fig. 1) no moths died within 24h ($n = 28$). Indeed all were alive after 48 h although 5 of 28 saline injected control females were dead at 48 h. This indicated that the toxic response was specifically associated with the presence of the pyrokinin/PBAN core (Phe-Thr-Pro-Arg-Leu-NH_2).

We do not know the mechanism of the toxicity of **2Abf-Suc**-Phe-Thr-Pro-Arg-Leu-NH_2. However, our results indicate that both the bromine and the pyrokinin/PBAN peptide core sequence are necessary for toxcisity. From this we hypothesize that the specific nature of the toxicity results from an interaction of the analog with receptor sites for the pyrokinin/PBAN class of insect neuropeptides. This class of neuropeptides has a wide range of known biological activities in insects including: hindgut and oviduct myotropic activity, pupariation, induction of egg diapause and reddish coloration and melanization, regulation of sex pheromone biosynthesis [15,17]. Additionally, neurohistochemical studies have shown that these peptides have a wide distribution within the insect nervous system. In moths, pyrokinin like immunoreactivity has been found in clusters of soma in the subesophageal ganglion (SEG), the thoracic ganglia and each of the segmental abdominal ganglia [3,5,6,9,10]. Axons extend from cells in the SEG to the corpora cardiaca as well as into the ventral nerve where they arborize in each segmental ganglion and terminate in the last abdominal ganglion. Axons containing pyrokinin immunoreactive peptides also extend from the SEG into the maxillary nerve and brain. Within the brain axons containing pyrokinin like immunoreactivity are concentrated in the ventral-median protocerebrum and along the edge of the mushroom bodies as well as in the antennal lobes and motor center. This indicates that pyrokinin/PBAN receptors are present in the brain and ventral nerve cord and that they control neural processes as well as other, as yet, undetermined neurophysiological events. Preliminary data using other amphiphilic analogs, including **9Fla**-Phe-Thr-Pro-Arg-Leu-NH_2, has indicated that these analogs are capable of penetrating the blood brain barrier of moths. Thus, it is possible that the amphiphilic character of **2Abf-Suc**-Phe-Thr-Pro-Arg-Leu-NH_2 enables the analog to penetrate the blood brain barrier and act on pyrokinin/PBAN receptors within the central nervous system, as well as at receptors in various non-neural tissues like the gut, oviduct, pheromone gland and, perhaps, even muscles.

The results demonstrate that a pseudopeptide analog of the pyrokinin/PBAN insect neuropeptide family, administered to a pest insect via injection can disrupt physiological processes to such an extent that 100% mortality is achieved. These toxic effects can be achieved at very low doses in adult females of the moth *H. virescens* (injected $EC_{50} = 0.7$ pmoles). The brominated pyrokinin/PBAN **2Abf-Suc**-Phe-Thr-Pro-Arg-Leu-NH_2 represents a lead analog for the potential future development of pest insect control agents based on this class of insect neuropeptides. Exposure of adult females, particularly those that have mated, to this or second generation analogs at bait stations could lead to a disruption of the efficiency of the propagation of moth species and, therefore, potentially to an overall reduction in pest populations.

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